Investigation on antimicrobial, antioxidant and cytotoxicity properties of triple bark extract formulated using traditional medicinal plants

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Abstract

Since ancient times, medicinal plants have been widely used against dreadful pathogens due to their ability to kill microbes. Also, the limitations of the present treatment methods have led the researchers to explore for new formulations with aspects of better wound healing and control of infections. In the present study antimicrobial, antioxidant and cytotoxicity properties of a polyherbal formulation prepared from the barks of three medicinal plants for treating wound infection were investigated. The polyherbal formulation was tested for broad spectrum antimicrobial activity against gram-positive bacteria Methicillin-Resistant *Staphylococcus aureus* (MRSA) and gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi* and antifungal activity against Fluconazole resistant *Candida albicans*. The result reveals that the polyherbal formulation exhibited antimicrobial activity against antibiotic resistant gram-positive MRSA and *C. albicans* and moderate against gram-negative *P. aeruginosa*, *E. coli* and *S. typhi*. The antioxidant activities like 1,1'-diphenyl-2-picrylhydrazyl and hydrogen peroxide scavenging ability of the formulation was compared with standard ascorbic acid. The higher antioxidant activity was found mostly due to the presence of higher amount of phenols and flavonoids. The phenol was found to be 48.63 ± 0.35 mg/g and flavonoids was 29.52 ± 1.13 mg/g. The polyherbal formulation helps in the proliferation of fibroblast cells without affecting the viability. The above result reveals that the formulation has good antimicrobial and antioxidant activity without affecting the viability of the cell.

Keywords Medicinal plants · Wound healing · Phenolics · Flavonoids · Cytotoxicity

1 Introduction

Alternative therapies are economical for wound healing with lesser side-effects and increases patient compatibility than the modern medicines [1]. The alternative therapies are based on the knowledge of traditional practices which includes siddha, ayurveda, unani, chinese and folk, etc. [2]. In such practices, herbs are commonly used as a core component of medicine. Traditional therapies mainly focus on the overall conditions of the individual rather than the particular ailment or disease [3]. The herbal medicines are better involved in disinfection, debridement and maintenance

of moist environment and helps to heal the wounds in natural way without any side effects [4].

Herbal medicines were used since pre-historic times, either alone or in combination with other components for wound healing [5]. They are in great demand due to their availability, less toxicity, less or no side effects [6]. About 163 species of plants are being used in the Indian system of medicine for wound healing [7, 8]. Most of the wound healing plants generally belong to various families namely Rubiaceae, *Euphorbiaceae*, *Moraceae* and *Acanthaceae* [9]. Generally, the herbal medicines are used either in the form of single herb (monoherbal) or combination of herbs (polyherbal). The phytochemical constituent of monoherbal

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formulation is minute and is insufficient to achieve desirable effect. Whereas, the polyherbal formulation shows the synergistic herb–herb interaction which enhances the therapeutic effect [10]. In addition, the multiple phytochemicals from polyherbal acts on multiple targets to enhance the pharmacological activity [11].

The polyherbal formulations are commonly used in Ayurveda and Siddha for the treatment of acute and chronic wounds. Honey and ghee are commonly used in Ayurveda whereas oil and ointment-based formulations are common in Siddha treatment [12]. Traditionally, the polyherbal formulations are prepared by mixing equal proportions of herbal powders and directly used as churna for the treatment of various internal and external diseases. Triphala, trikadu and sudharsana are some of the ayurvedic formulations prepared by mixing equal proportions of herbal powders [13]. Likewise, this study is involved in the preparation of triple bark formulation by mixing equal proportions of bark powders such as *Acacia catechu*, *Azadirachta indica* and *Syzgium cumini*. *A. catechu* (*Fabaceae*) is one of the most commonly used plants in traditional medicine for its antimicrobial, antioxidant, astringent, anti-ulcer and wound healing activities. This is mainly due to the presence of various important factors such as flavonoids, tannins, glycosides and sugars. Among various parts, bark and heartwood are the most commonly used parts for various ailments since centuries [14, 15]. The *A. catechu* bark is used in the treatment of wound for its astringent property [16]. *Azadirachta indica* (*Meliaceae*) generally considered as ‘village dispensary’ and nature’s gift is used in the treatment of various skin diseases. It is used for its anti-inflammatory, immunomodulatory and antibacterial activity. Terpenoids is one of the main active constituents of neem along with diterpenes, phenols and polysaccharides [17]. *Syzgium cumini* (*Myrtaceae*) is mainly used for the treatment of diabetes and is responsible for hypoglycemic, immunomodulatory and anti-infective properties. Phytochemicals like sterols, resin, tannins, phytosterol and triterpenoids are common in *S. cumini* [18].

The aim of the study is to prepare a triple bark polyherbal formulation followed by investigations of antimicrobial, antioxidant and cell proliferation ability of the formulation at in vitro conditions. This study also includes qualitative and quantitative phytochemical analysis of the crude bark extract.

2 Materials and methods

2.1 Herbal collection and extract preparation

The Stem barks of *A. catechu*, *A. indica* and *S. cumini* were purchased from the herbal shop, India. The herbal materials were authenticated by a botanist from Centre for

Advanced Studies in Botany, Madras University. The barks were washed, shade dried and powdered using laboratory blender and the powders were sieved through 40 mesh sieve to get fine powders. The powders were mixed at equal proportions and extracted using soxhlet apparatus with 70% methanol. The solvent was removed using rotavapour evaporator and the extract was collected and stored at 4 °C.

2.2 Microorganisms

Pure cultures of Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi* and fluconazole resistant *C. albicans* were used to test the antimicrobial activity of the formulation. All the bacterial and fungal pathogens are clinical isolates maintained in the Centre for Advanced Studies in Botany, Madras University.

2.3 Phytochemical analysis

The phytochemicals of triple bark formulation were analyzed by standard procedures [19–21] and was compared with the monoherbals. The phytochemical markers like phenolics and flavonoids of polyherbal extract were estimated by Folin’s phenol method and Aluminium chloride method respectively [22]. The phenolic content was expressed in mg GAE (Gallic Acid Equivalent)/g of dry extract and the flavonoids in mg QE (Quercetin Equivalent)/g of dry extract.

2.4 Antioxidant activity study

The antioxidant activity was based on the hydrogen donating or free radical scavenging ability of the phytochemicals present in the extract. DPPH (1, 1'-diphenyl-2-picrylhydrazyl) and hydrogen peroxide scavenging methods were used to analyze the antioxidant potential of the bark formulation [23].

2.5 Antimicrobial study

2.5.1 Agar well plate method

The antimicrobial activity was performed based on the modified Kirby–Bauer method. 50 µL of samples at the concentration ranging from 5%, 10% and 15% were loaded in the Mueller–Hinton agar plates and the plates were incubated at 37 °C for 24 h. Streptomycin sulphate and 20% DMSO (dimethyl sulfoxide) were used as positive and negative controls respectively for test pathogens. The zone of inhibition was measured and the results were tabulated as mean ± SD.

2.5.2 Determination of minimum inhibitory concentration (MIC)

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay is commonly used in recent days for MIC determination as it is simple and highly sensitive compared to micro-dilution assay. For this study, 100 μ L of Muller Hinton broth was dispensed into a 96 well micro titer plate and the samples were loaded at the concentration of 10–100 μ g/mL. To this, 20 μ L of inoculums of selected bacteria at the concentration of 1×10^5 CFU/mL was added and the plates were incubated at 37 °C in aerobic condition for 18 h. After incubation, 15 μ L of 5 mg/mL concentration of MTT solution was added and further incubated for 4 h at 37 °C for the development of formazan crystals. The supernatant was removed and replaced with DMSO to dissolve formazan crystals. The lowest concentration at which the formazan crystals were not formed is considered to be the minimum inhibitory concentration (MIC) for the pathogens [24].

2.6 Cell viability assay

The cytocompatibility of the polyherbal formulation was studied on mouse 3T3 fibroblast cells using MTT assay. The fibroblast cells were inoculated in 25 cm² flask with DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine serum) and 1% penicillin–streptomycin and incubated at 37 °C at 5% CO₂ atmosphere. The cells were allowed to form 90% confluent monolayer and washed with phosphate buffer followed by 5 mL of trypsin–EDTA treatment to dissolve the monolayer. After adding fresh DMEM medium, 100 μ L of cells (1×10^6 cells/well) were transferred to the 96 well plates and incubated at 5 °C for 24 h at 5% CO₂ atmospheric condition. To this, 100 μ L of media containing 50 and 100 μ g/mL of extract was added and incubated. After 24 h and 48 h of incubation, the media was removed and 15 μ L of 5 mg/mL concentration of MTT solution was added and incubated for 4 h. The plates were read at 570 nm in the micro plate reader [24].

2.7 Statistical analysis

The experiments were performed in triplicates and the values were expressed in mean $\pm$ SD.

3 Result and discussion

3.1 Extraction and phytochemical investigation

The soxhlet method using methanol yielded 10.29 ± 0.24 g of polyherbal extract per 100 g of triple bark powder. The

extraction yield depends on the type of solvent and the method of extraction used [25]. Soxhlet method is the commonly and conveniently used method for natural product isolation which is thermostable in nature [26] and is considered inexpensive due to less solvent usage [27]. The solvent selection is another important factor in isolating different phytochemicals from the herbal. The organic solvent is considered good for the extraction of phenolics and flavonoids. Methanol is considered as highly polar solvent for the extraction and separation of a wide range of components present in the medicinal plants [28]. In addition, it is of less cost and free from ethical issues compared to ethanol [29]. The results of previous studies have also proved the efficiency of methanol in extracting the antimicrobial principle [30]. Hence, in this study we have used soxhlet apparatus and methanol for extraction.

The results of the qualitative phytochemical analysis are shown in Table 1 and the result shows the presence of phenols, flavonoids and tannins followed by saponins, triterpenes, steroids, glycosides, carbohydrates, proteins and amino acids. The results of phytochemical analysis were compared with the methanolic extracts of mono-herbal to identify the specific constituents in the selected herbs. Phenols, Tannins and flavonoids are common in all the three barks. Saponins were found in the *A. catechu* and *A. indica* but not in *S. cumini*. Carbohydrates and reducing sugars are from *A. indica* and the amino acids are from *S. cumini* but there are no proteins and amino acids in *A. catechu*.

The marker compounds like phenolics and flavonoids are considered as common phytochemicals of antimicrobial and antioxidant activity other than active constituents of herbs. The results of the total phenol and flavonoid

Table 1 Phytochemical analysis of triple bark extract and mono-herbals

Test methods	Triple bark extract	<i>Acacia catechu</i>	<i>Azadirachta indica</i>	<i>Syzgium cumini</i>
Phenols	+++	+	+	+
Flavonoids	++	++	+	+
Tannins	+++	+	+	+
Terpenoids	++	–	++	+
Saponins	++	+	++	–
Glycosides	+	+	–	+
Steroids	+	–	–	+
Carbohydrates	+	+	+	–
Proteins	+	–	+	–
Sugars	+	+	+	+
Amino acids	+	–	+	+

+++ High concentration, ++ medium concentration, + low concentration, – absence

content was found to be 48.63 ± 0.35 mg GAE/g dry weight and 29.52 ± 1.13 mg QE/g of the crude extract respectively. The result shows that the formulation has higher concentration of phenols and flavonoids. The higher amount of phenols in the formulation is due to the presence of gallic acid and gallic acid derived tannins from *A. indica* [31], *A. catechu* [32] and *S. cumini* [18]. The phenolics are responsible for the antibacterial and antifungal activity of the formulation. In addition, the presence of higher flavonoid contents such as catechin, epicatechin, quercetin, kaempferol and myricetin in the formulation are considered as good agent for antioxidant activity which is similar to that of standard ascorbic acid. The antioxidant activity of flavonoids is due to the stabilization of the free radicals through hydrogenation or complexing with oxidizing species. This is one of the important properties for wound healing formulation to prevent tissue damage caused by oxidants, developed during inflammation, immune action and infection at the wound site [33].

The results of the DPPH and H_2O_2 radical scavenging activity were found to be akin to the standard ascorbic acid. At low concentration, the formulation shows less DPPH scavenging activity, but at 100 $\mu\text{g/mL}$ the PHF reduces 91% of DPPH which is comparable with ascorbic acid (94%) as shown in Fig. 1. But, the hydrogen peroxide scavenging ability of extract was found to be higher than the ascorbic acid as shown in Fig. 2. The IC_{50} value for DPPH and H_2O_2 radical scavenging activity of extract and ascorbic acid is given in Table 2.

The results of antimicrobial activity of the extract are shown in Fig. 3. The results show that the polyherbal extract is highly active against the gram-positive bacteria *S. aureus* and the fungi *C. albicans*. The extract was found to show moderate activity towards gram negative bacteria *P. aeruginosa*, *E. coli*, *S. typhi*. From the results obtained, it can be concluded that the polyherbal extract has

Fig. 1 DPPH scavenging ability of ascorbic acid and extract

broad-spectrum antimicrobial activity against the tested pathogens. The result reveals the higher activity of extract against gram positive and fungal cellwall. The moderate activity of the extract against gram negative bacteria was due to inherent tolerance of the bacteria. The presence of lipopolysaccharide layer and periplasmic space of the gram-negative bacteria is responsible for the resistance of the bacteria [34]. The results of MIC value determined by MTT assay are summarized in Table 3. The MIC of the extract was found to be lesser for MRSA, *S. typhi*, *P. aeruginosa* and moderate for *C. albicans* and higher for *E. coli*.

The bacterial resistance is one of the problems in treating infection with antibiotics. The resistance developed is also due to over-prescription of antibiotics which leads to development of superbugs such as MRSA (*Methicillin Resistant Staphylococcus aureus*) and VRE (*Vancomycin Resistant Enterococci*). The staphylococcal or MRSA infection makes the body vulnerable to secondary infection with yeast (*Candida*) and fungi during surgery or weakened immune system. The secondary infections are hard to treat and lead to chronic condition. In recent past, the MRSA infection has been found to be predominant in diabetic ulcers [35]. The multiple antimicrobial components present in the polyherbal extract acts on multiple targets thereby helping to prevent the development of resistance on the bacterial cells. The triple bark formulation is

Fig. 2 H_2O_2 scavenging ability of ascorbic acid and extract

Table 2 The IC_{50} value for antioxidant activity of ascorbic acid and the formulation

Assay Method	Ascorbic acid ($\mu\text{g/mL}$)	Herbal formulation ($\mu\text{g/mL}$)
DPPH	15.52 ± 0.26	29.03 ± 0.75
H_2O_2	20.88 ± 0.57	19.81 ± 2.7

Mean $\pm$ standard deviation, $n = 3$

Fig. 3 Antimicrobial activity of triple bark formulation. The values are mean $\pm$ SD, n=3; *SS Streptomycin sulphate

The values are mean $\pm$ SD, n=3; *SS-Streptomycin Sulphate

Table 3 MIC of triple bark formulation by MTT assay

S. no.	Microbes	MIC value (μ g/mL)
1	<i>Salmonella typhi</i>	40
2	<i>Pseudomonas aeruginosa</i>	50
3	MRSA	30
4	<i>Escherichia coli</i>	50
5	<i>Candida albicans</i>	30

MRSA Methicillin resistant *Staphylococcus aureus*

considered to be a good antimicrobial agent for the treatment of bacterial, fungal, diabetic foot ulcers and other secondary infections. The mechanism of antimicrobial action varies based on the presence of phytochemical constituents. The phytochemicals like flavanols, flavonols and tannins may be responsible for the wide spectrum antimicrobial activity of the polyherbal formulation. Specifically, flavonols such as quercetin, kampherol and myricetin of the formulation showed broad spectrum activity due to hydrophobicity and membrane penetrating ability exerted inside the bacteria. The tannins act by getting mixed with membrane proteins through non-covalent interactions. The mechanism of antimicrobial activity also involves the destabilization of cytoplasmic membrane, permeabilization of cell membrane and inhibition of extracellular enzymes. Tannins directly affects microbial metabolism or prevents substrate utilization and thereby limits the bacterial growth [36].

The results of the MTT assay that represent the cell viability or cell proliferation of 3T3 fibroblast cells after 24 h of incubation is shown in Fig. 4. Generally, the results are shown only up to 100 μ g/mL. The increasing concentration decreases the viability of cells and the IC₅₀ value of the extract is found to be 274.30 μ g/mL. The absorption values of control cells are adjusted to 100% viability. Cytotoxicity of the sample is rated on the basis of the percentage viability relative to control group. The samples are

The data are presented as mean $\pm$ Standard deviation of three replicates.

Fig. 4 Percentage viability of 3T3 fibroblast cells. The data is presented in mean $\pm$ standard deviation of three replicates

considered non-toxic, if the viability is > 90%. The value between 60–90% viability indicates a slight toxicity and 30–59% means a moderate toxicity. The values < 30% indicates that the sample is severely toxic to the cells. In the present study the IC₅₀ value was found to be 100 μ g/ μ L [37].

4 Conclusion

The polyherbal formulation prepared by mixing the barks of three different medicinally valuable plants was found to show good antimicrobial and antioxidant activity. The presence of different phytochemicals from different plants was considered responsible for the antimicrobial and antioxidant activity of the formulation. The polyherbal extract showed good antimicrobial activity against gram positive and moderate towards gram-negative bacteria. The results showed that this formulation can be used to prevent hospital acquired infection caused by MRSA and a

fungi *C. albicans*. This antimicrobial property can be helpful in controlling and preventing the wound from infection. The antioxidant property was found to be responsible for scavenging free radicals and thereby reducing the risk of tissue damage at the wound site. The cell proliferation efficiency up to 100 µg/mL shows the good compatibility of the material, as it is another major drawback in chronic wounds. From all the above results, it is concluded that the triple-bark polyherbal extract can be a promising candidate for the treatment of infected chronic wounds. The further study in this direction is required to identify the wound healing ability of this formulation in in vivo model. This will also be useful for the dermatological applications to control infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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